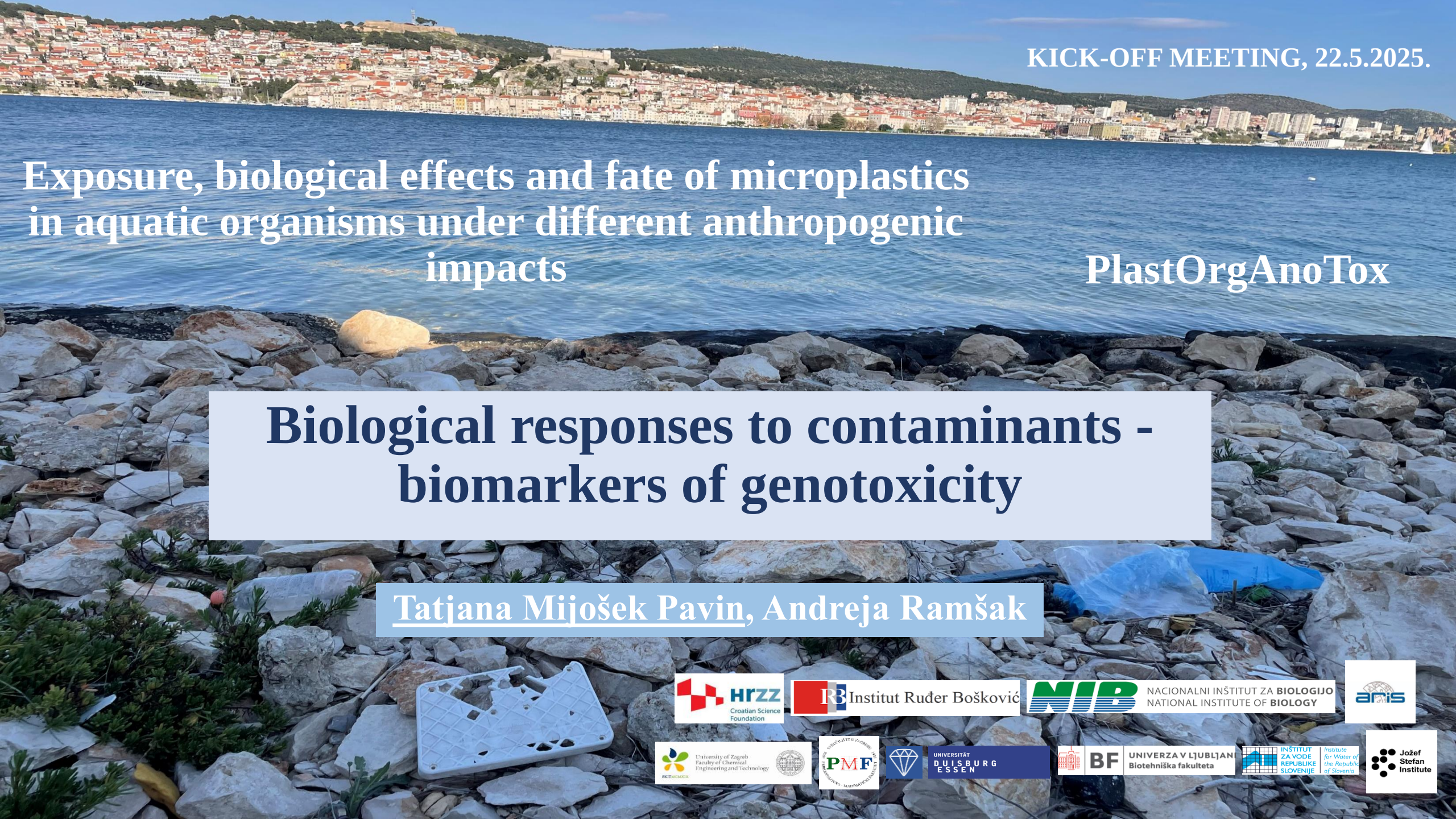


KICK-OFF MEETING, 22.5.2025.

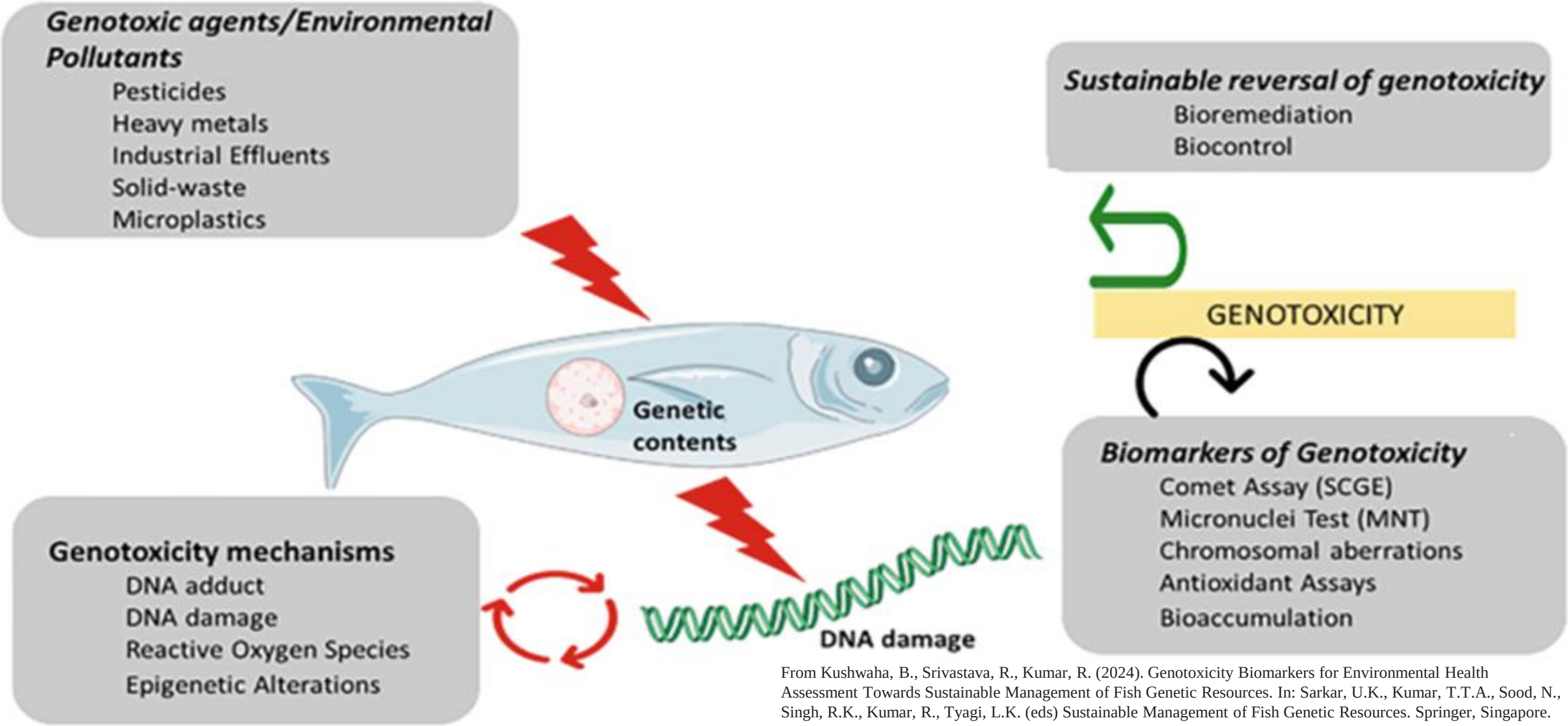
Exposure, biological effects and fate of microplastics in aquatic organisms under different anthropogenic impacts

PlastOrgAnoTox

Biological responses to contaminants - biomarkers of genotoxicity

Tatjana Mijošek Pavin, Andreja Ramšak

Genotoxicity refers to the capability of a substance to damage the genetic information of cells (changes of the structure, sequence, and/or number of genes), which can have direct or indirect effects.

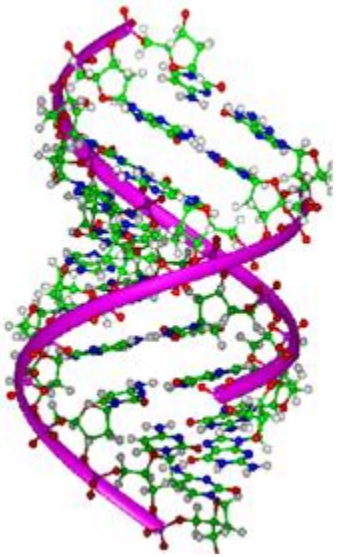


From Kushwaha, B., Srivastava, R., Kumar, R. (2024). Genotoxicity Biomarkers for Environmental Health Assessment Towards Sustainable Management of Fish Genetic Resources. In: Sarkar, U.K., Kumar, T.T.A., Sood, N., Singh, R.K., Kumar, R., Tyagi, L.K. (eds) Sustainable Management of Fish Genetic Resources. Springer, Singapore.

Comet test

- Microgel electrophoresis of individual cells – fragmented DNA migrates toward the anode, forming a comet-like appearance with visible "tail,, - the longer the tail, the more damage present. Undamaged DNA remains in the "head" of the comet.
- Developed by Östling and Johanson in 1984 (neutral comet)
- Modified by Singh in 1988 (alkaline comet)

Detectable types of damage:



DNA breaks (up to several hundred breaks per cell)

alkali-labile sites (apurine and apyrimidinic (AP) sites)

DNA-DNA and DNA-protein cross-links

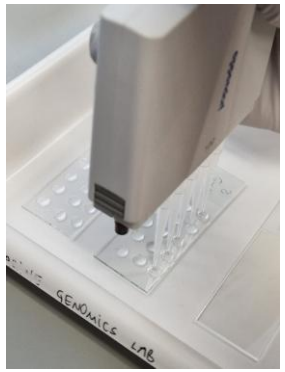
Depending on pH values different DNA damage detected

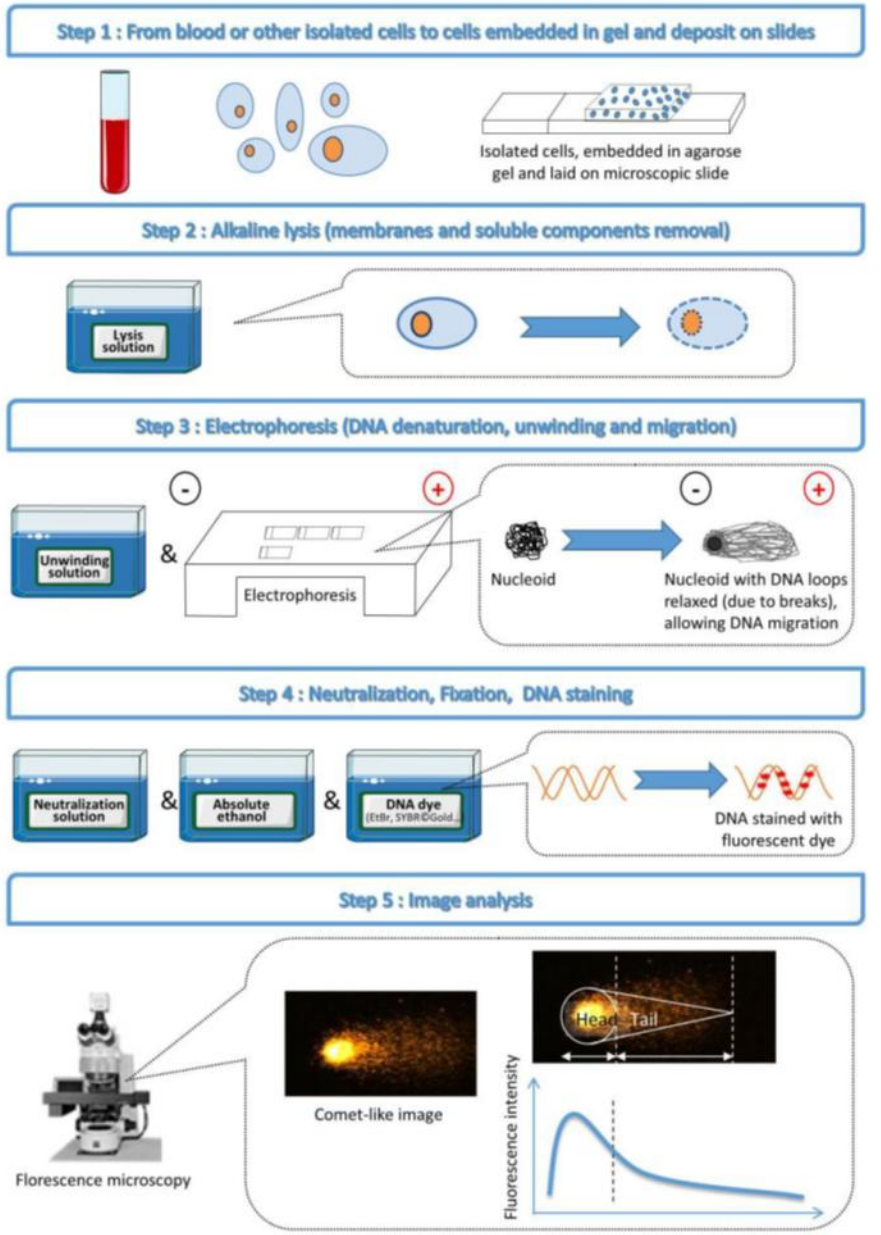
A neutral comet assay, performed at a neutral pH (7-9), primarily detects double-strand breaks (DSBs).
An alkaline comet assay, conducted at a pH ≥13, is more sensitive and can detect both DSBs and single-strand breaks (SSBs), as well as alkali-labile sites.

Single-strand breaks - the most common primary DNA damage = the most sensitive indicator of DNA damage

Comet test protocol

- Embedding in a agarose gel – 1% LMP
- Cell lysis - Solution at pH 10 with NaCl, EDTA and Triton X-100 – at least one hour!
- DNA denaturation - Solution with 10M NaOH + Na_2 EDTA at pH 13
- Electrophoresis
- Neutralization – Tris-HCl buffer, pH 7.5
- Fixation - ethanol
- Staining – AO/EtBr
- Image analysis – fluorescence microscopy





Big thanks to Margareta Kračun Kolarević and Stoimir Kolarević from Institute for Biological Research "Siniša Stanković„, National Institute of the Republic of Serbia, University of Belgrade

From Azqueta, A., Ladeira, C., Giovannelli, L., Boutet-Robinet, E., Bonassi, S., Neri, M., Gajski, G., Duthie, S., Del Bo', C., Riso, P., Koppen, G., Basaran, N., Collins, A., & Møller, P. (2020). Application of the comet assay in human biomonitoring: An hCOMET perspective. *Mutation research. Reviews in mutation research*, *783*, 108288.

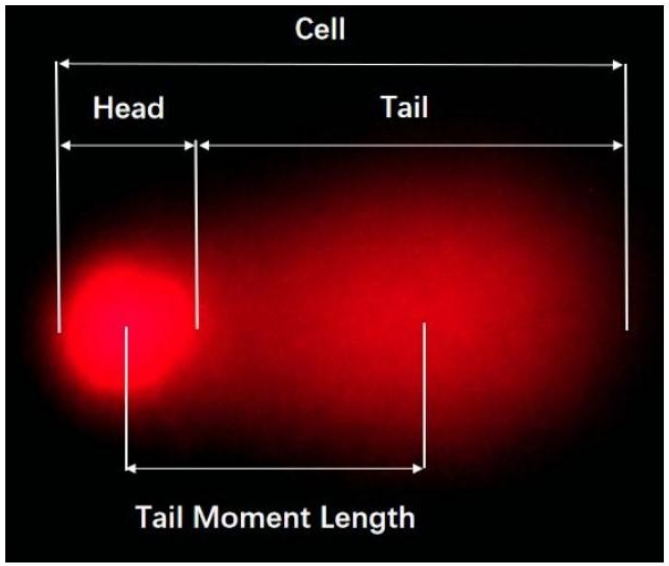
DNA damage in comet assay is evaluated based on the proportion of DNA in the “tail” and “head” of the comet.

Measurements are made using softwares for image analysis which analyzes multiple descriptors:

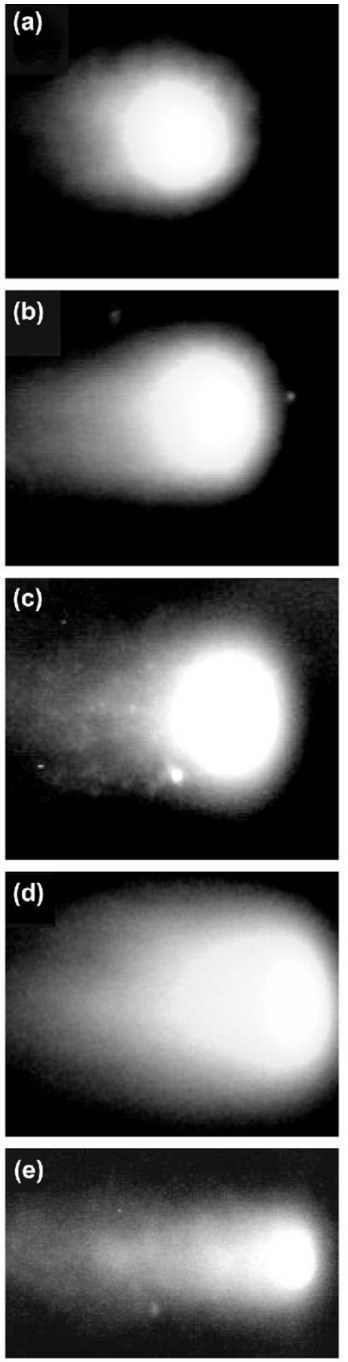
1. **Tail length-** distance to which DNA fragments migrated during electrophoresis (proportional to DNA damage)

2. **% DNA in tail** – determined by image analysis, indicates amount of damaged DNA

3. **Tail moment** – calculated by the formula: tail length × % DNA in tail / 100



From Linhartova, P., Gazo, I., Shaliutina, A., & Hulak, M. (2013). The in vitro effect of duroquinone on functional competence, genomic integrity, and oxidative stress indices of sterlet (Acipenser ruthenus) spermatozoa. Toxicology in vitro : an international journal published in association with BIBRA, 27(6), 1612–1619.



Advantages of comet test

- Does not require mitotically active cells
- Applicable to any eukaryotic cell type
- Small number of cells required to perform the test
- Small sample volume needed
- Detects damage in individual cell
- High sensitivity
- Simplicity
- Relatively low cost
- Rapid test
- Wide application

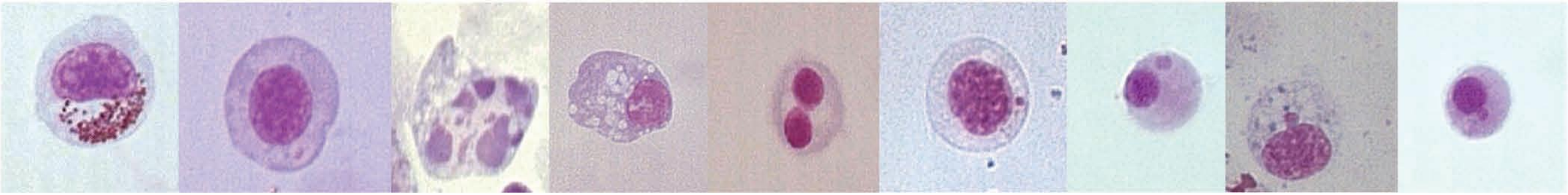
Micronucleus test

- Micronucleus – small independent extra nucleus in the cytoplasm containing chromosome fragments or whole chromosomes, which failed to incorporate in the main nucleus during cell division, and that is completely separate from the nucleus.
- Originally developed with mammalian species but is today widely applied in fish and other aquatic organisms.

- Target tissues:

1. Mussels - Hemocytes and gill cells are the targets tissues most frequently considered for the evaluation of micronuclei frequency. The hemolymph provides easily collectable single-cell suspension, but its main limitation for the application of micronucleus test is the complexity of cell types. Different subpopulations, including granular and agranular cells were described in the hemolymph with different origin and functions not completely known. Gills represent an ideal target for the evaluation of the micronuclei frequency showing a more sensitive response to genotoxic agents compared with hemocytes. The cell suspension from gills is heterogeneous in composition including larger epithelial cell types with or without cilia, and with large cytoplasm and well-spread nuclear chromatin, as well as some smaller cells with a higher nucleus/cytoplasm area ratio. Evaluation of only the **agranular cells** in both the target tissues is strongly suggested for the MN scoring, in order to avoid the confounding factors represented by the cytoplasmic granules.
2. Fish - Micronuclei in fish can be visualized in different cell types such as gill, kidney, hepatic cells although the use of **peripheral erythrocytes** is more widespread because it avoids the complex procedures of cell preparation and the killing of animals.

Cell types in haemocytes of mussels:



different cell types stained using 3% (vol/vol) Giemsa. (**a**) Granular hemocyte. (**b**) Agranular hemocyte. (**c**) Apoptotic agranular hemocyte. (**d**) Necrotic agranular hemocyte. (**e**) Binucleated agranular hemocyte. (**f**,**g**) Agranular hemocytes with MNi. (**h**,**i**) Agranular hemocytes with buds.

PROTOCOL

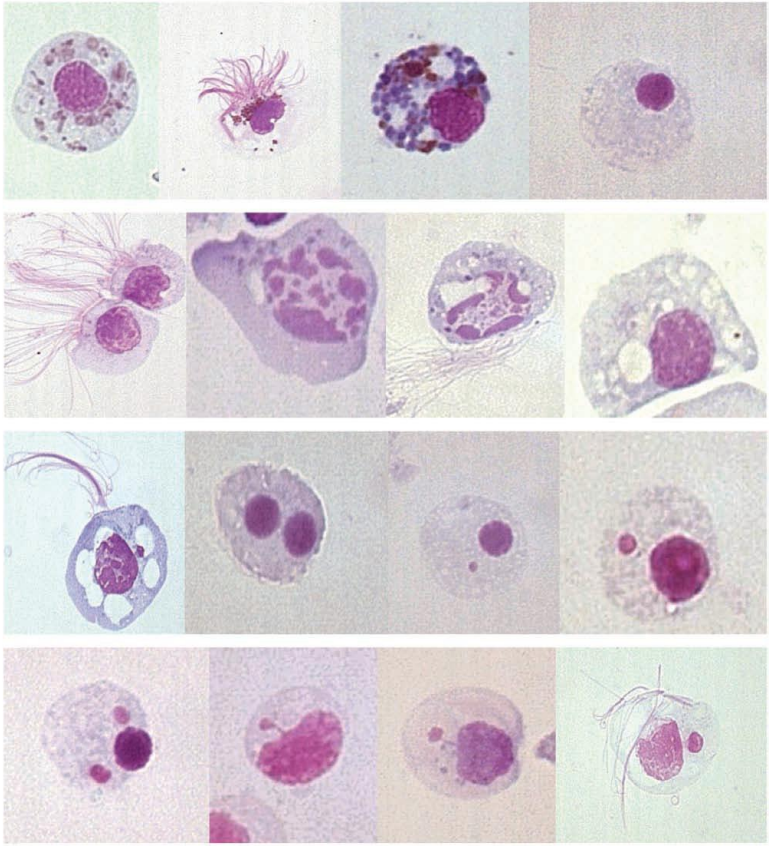
Mussel micronucleus cytome assay

Claudia Bolognesi[1] & Michael Fenech[2]

[1]Environmental Carcinogenesis Unit, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Azienda Ospedaliera Universitaria San Martino–IST, Istituto Nazionale Ricerca sul Cancro, Genova, Italy. [2]Commonwealth Scientific and Industrial Research Organization (CSIRO) Food and Nutritional Sciences, Adelaide, South Australia, Australia. Correspondence should be addressed to C.B. (claudia.bolognesi@istge.it).

Detailed description and protocol in Bolognesi, C., & Fenech, M. (2012). Mussel micronucleus cytome assay. Nature protocols, 7(6), 1125–1137.

Haemolymph – sampling, preparing slides with the sample and drying, fixation, dying, counting
Gills – sampling, enzymatic degradation, two-step filtration of suspension, centrifugation, resuspending pelet with fixative, preparing slides with the sample, drying, dying, counting



Cell types in gills of mussels:

different cell types stained using 3% (vol/vol) Giemsa. (a) Granular epithelial aciliated cell. (b) Granular epithelial ciliated cell. (c) Granular hemocyte. (d) Agranular epithelial aciliated cell. (e) Agranular epithelial ciliated cell. (f) Apoptotic agranular epithelial aciliated cell. (g) Apoptotic agranular epithelial ciliated cell. (h) Necrotic agranular epithelial aciliated cell. (i) Necrotic agranular epithelial ciliated cell with a micronucleus. (j) Binucleated agranular epithelial aciliated cell. (k,l) Agranular epithelial aciliated cells with MNi. (m) Agranular epithelial aciliated cells with two MNi. (n–p) Agranular epithelial aciliated cells with buds.

Types of damage that are counted:

Micronuclei = originate from acentric chromosome fragments or entire chromosomes left behind in anaphase, and their presence is an indicator of the existence of aberrations that occurred in the previous cell division and are used as a measure of structural and numerical chromosome aberrations.

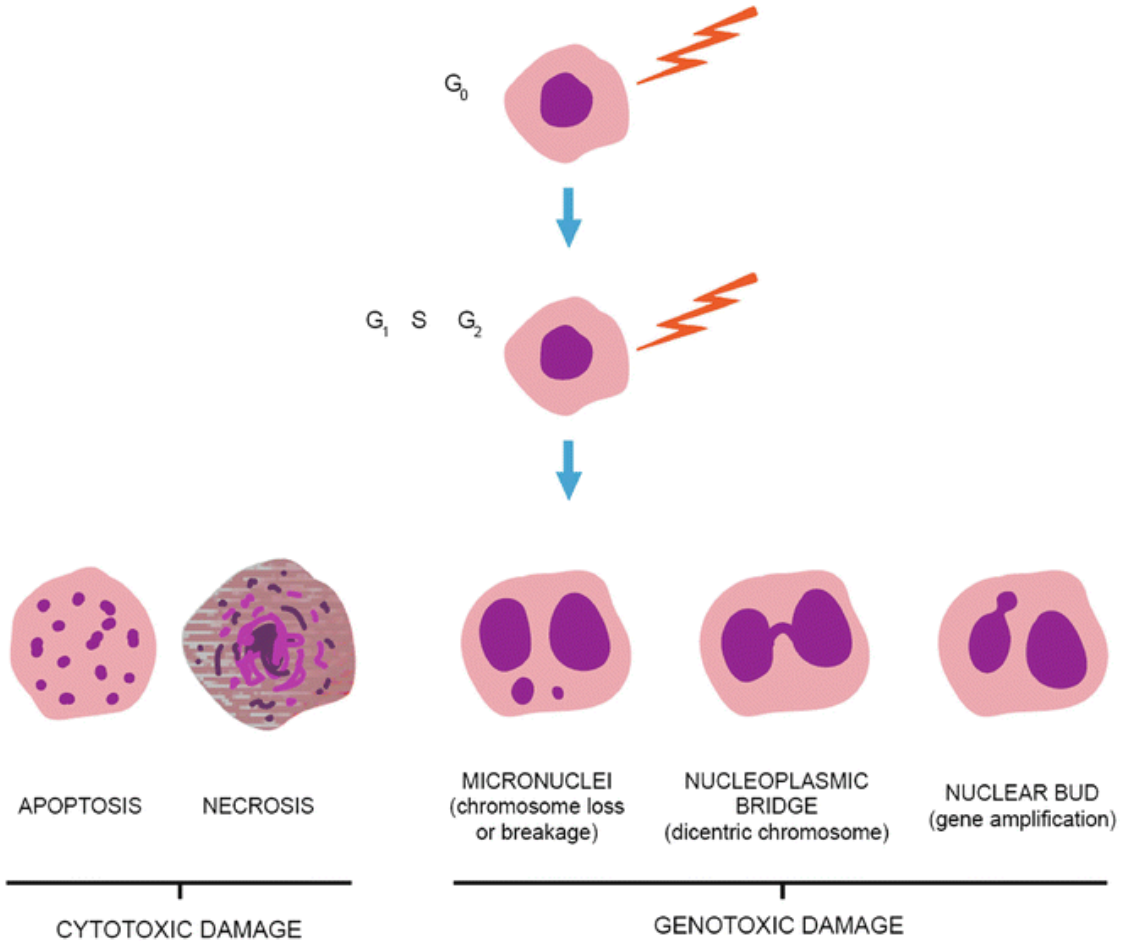
Nuclear bridges = arise as a consequence of the formation of dicentric chromosomes, in which the centromeres are pulled to opposite poles of the cell.

Nuclear buds = arise as a consequence of gene amplification, which results in the expulsion of the amplified region into the nuclear bud.

Slide scoring - light microscopy at ×1,000 magnification and score at least 1,000 cells per slide, but can be automated; triplicate per each individiual is made.

Criteria for determining micronuclei:

- **MN diameter less than 1/3 of the nucleus**
- **Oval or round shape**
- **Not connected to the nucleus**
- **MN color equal or slighlty lighter from the color of the nucleus**



From Migliore, L., Di Bucchianico, S., Uboldi, C. (2014). The In Vitro Micronucleus Assay and FISH Analysis. In: Sierra, L., Gaivão, I. (eds) Genotoxicity and DNA Repair. Methods in Pharmacology and Toxicology. Humana Press, New York, NY.

Advantages of MN test

- Applicable to many cell types
- High sensitivity
- Simplicity
- Relatively low cost
- Wide application
- Useful for long-term or cumulative damage assessment

Summary

Feature	Comet Assay	Micronucleus Test
DNA damage type	Strand breaks	Chromosome break/loss
Tissue types	Blood, gills, hemocytes	Blood, gills, hemocytes
Sensitivity	Very high	Moderate
Reversibility	**Often reversible**	**Usually irreversible**
Time to damage detection	**Short-term exposure**	**Longer-term/cumulative exposure**
Quantification	Continuous (image analysis)	Discrete (count-based)

Both tests have strengths and limitations, but applying both tests as complementary methods to different target tissues (hemolmyph/blood and gill cells) from the same animals is suggested to have a more complete picture of the genotoxic effects induced by the cumulative exposure to the mixture of contaminants in the studied areas in nature or during the exposure experiments in the laboratory.

Genotoxicitiy testing in frame of PlastOrgAnc

- Tests: Comet and Micronucleus test
- Biondicators: mussels and fish
- Fish blood and gills fixated and stored after the field




Preparation at MBP:

Thanks to MBP team for introducing us with the methods!

Thanks to Margareta and Stoimir for the comet protocol and scoring the slides!

Thanks for listening!

Fish:

- Protocols will need to be adjusted
- Possible effects of freezing? Used in literature, so the effect should n